FEBS Letters 579 (2005) 554–558 FEBS 29152

Deletion analysis in the catalytic region of the 10–23 DNA enzyme

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Received 23 November 2004; revised 24 November 2004; accepted 1 December 2004

Available online 18 December 2004

Edited by Hans Eklund

Abstract In this study, the functional relevance of the core nucleotides of the RNA cleaving 10–23 DNA enzyme (DNAzyme) was investigated. Systematic deletion studies revealed that DNAzymes lacking thymine at position 8 (T8) retain catalytic activity comparable to that of the wild-type enzyme. Deletion of the adjacent cytosine at position 7 (C7) also resulted in a highly active enzyme and even the double deletion mutant C7/T8 displayed cleavage activity, although the catalytic rate under multiple turnover conditions was found to be reduced by one order of magnitude. The identification of non-essential nucleotides in the catalytic core might help to stabilize the DNAzyme against nucleolytic degradation and to overcome problems in elucidating its three-dimensional structure.

Keywords: DNA enzyme; DNAzyme; Ribozyme

1. Introduction

RNA cleaving DNA enzyme (DNAzymes) are valuable tools with potential applications in functional genomics and gene therapy. The most prominent representative of this class of catalytically active nucleic acids is the 10–23 DNAzyme (Fig. 1), which was obtained by in vitro selection from a combinatorial library [1]. This DNAzyme consists of a catalytic region of 15 nucleotides and two flanking arms with 6–12 nucleotides each that bind to the complementary target RNA substrate via Watson–Crick base-pairing. It catalyzes Mg^{2+}-dependent cleavage between an unpaired purine and a paired pyrimidine [2].

The 10–23 DNAzyme has been used successfully to suppress the expression of viral or endogenous target genes in vitro and in vivo [3,4]. For example, HIV infection of cells and virus replication were inhibited by DNAzymes targeting either viral genes [5,6] or the cellular co-receptor CCR5 [7]. The DNAzyme has also been used to inhibit smooth muscle cell proliferation [8] and in the first in vivo study, a DNAzyme targeting the mRNA of early growth response factor-1 inhibited neointima formation after balloon injury to the rat carotid artery wall [9].

Despite a number of successful applications, the knowledge about cleavage mechanism and three-dimensional structure of the 10–23 DNAzyme is limited compared to the information that has been obtained for the hammerhead ribozyme. Kinetic measurements of the DNAzyme were performed to analyze the influence of pH, the choice of divalent metal cofactor and its concentration on the cleavage activity [10]. We have recently performed a functional analysis of sequence requirements within the catalytic region of the 10–23 DNAzyme by replacing individual bases by naturally occurring or modified nucleotides [11]. In the present study, we investigated the importance of each residue within catalytic core of the DNAzyme by deletion of each single nucleotide and analysis of the remaining activity of the mutant DNAzymes. Two DNAzymes were employed that were recently identified to cleave the mRNA of the vanilloid receptor subtype 1 (VR1) with high efficiency [12] and a third DNAzyme targeting the 5′ non-translated region of the human rhinovirus-14 was used as an independent control.

2. Materials and methods

2.1. Oligonucleotides

All DNA oligonucleotides were obtained from MWG Biotech, Ebersberg, Germany. PAGE purified RNA targets were purchased from IBA GmbH, Göttingen, Germany. Sequences of the DNAzymes and their RNA substrates are summarized in Table 1.

2.2. Determination of cleavage activity of DNAzymes under single turnover conditions

For an initial screening, cleavage activity of DNAzymes with single nucleotide deletions in the catalytic core was determined under single turnover conditions. The 1-pmol unlabeled and 20 000 cpm radioactively labeled short target RNA (19-mer) were mixed and used for the cleavage reaction in ribozyme buffer (10 mM $MgCl_2$, 50 mM Tris–HCl, pH 7.5) at 37 °C for 20 min with a 10-fold excess of the DNAzyme. Substrate RNA and cleavage products were separated by PAGE. Radioactive signals were detected with a Molecular Dynamics Storm 840 Phosphoimager and the density of radioactive bands was quantified by the program ImageQuaNT.

For experiments with a long substrate, VR1 mRNA (2614 nucleotides) was transcribed with T7 RNA polymerase [12]. In initial experiments, a 10-fold excess of DNAzyme in ribozyme buffer was used. For analysis of ion dependency of the DNAzymes, cleavage was measured at 37 °C with a saturating 100-fold excess of DNAzyme in ribozyme buffer containing different ions (Mg^{2+} and Ca^{2+}) at concentrations ranging from 1 to 10 mM. Aliquots were taken at appropriate time points and cleavage was analyzed by agarose gel electrophoresis. Bands were quantified using the Quantity One program (Bio-Rad, Munich, Germany) and data were analyzed by fitting with a single exponential decay function using the Microcal Origin program. Mean values and standard deviations of three independent experiments are given.

2.3. Multiple turnover kinetic analysis with the short target RNA

In multiple turnover kinetic analysis, varying concentrations of the radioactively labeled short target RNA ranging from 10 to 1000 nM were cleaved by 1 nM of the DNAzyme. DNAzymes and RNA targets were denatured separately and after renaturation the reaction was started by adding the DNAzyme to its RNA substrate. Aliquots were

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Abbreviation: VR1, vanilloid receptor subtype 1

0014-5793/$22.00
doi:10.1016/j.febslet.2004.12.008

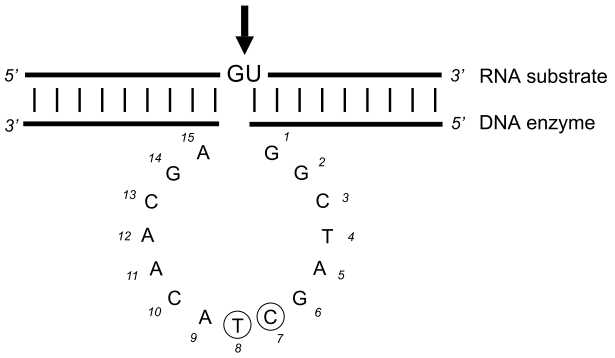

Fig. 1. Secondary structure of the 10–23 DNAzyme. The arrow indicates the cleavage site. Nucleotides that can be deleted without severe impact on the catalytic activity are marked with circles.

taken at appropriate time points and the reaction was stopped by the addition of loading buffer (90% deionized formamide, 20 nM EDTA and 0.05% bromophenol blue/xylencyanole) and freezing the sample in liquid nitrogen. Cleavage reactions were analyzed by PAGE. Initial velocities of each experiment were determined by linear fitting of the first 10% of the cleavage reaction with the Microcal Origin program (Northampton, MA). K_M and k_{cat} values were obtained by plotting initial velocities as a function of substrate concentration and hyperbolic fitting of the data. Mean values and standard deviations of three independent experiments are given.

Table 1
Sequences of DNAzymes (DH5, DV15 and DV29) and their RNA targets

DH5	5′ CCG GGG AAA GGC TAG CTA CAA CGA AGA AGT GCT 3′
RNA target for DH5	5′ AGC ACU UCU UUU CCC CGG 3′
DV15	5′ ATG TCA TGA GGC TAG CTA CAA CGA GGT TAG GGG 3′
RNA target for DV15	5′ CCC CUA ACC GUC AUG ACA U 3′
DV29	5′ TCT TGT TGA GGC TAG CTA CAA CGA GGT CTC ACC 3′
RNA target for DV29	5′ GGU GAG ACC GUC AAC AAG A 3′

DH5 targets the 5′ non-translated region of the human rhinovirus, DV15 and DV29 are directed against sequences of the VR1 mRNA. The catalytic region of the DNAzyme is underlined.

2.4. Melting curves

Melting temperatures were determined for duplexes of DNAzymes and their 19-mer target RNA with a HP-Spectrophotometer 8452A. Measurements were performed at a duplex concentration of 1.5 μM in ribozyme buffer over a temperature range of 20–90 °C. In order to avoid substrate cleavage, the RNA target molecule was modified by attaching a 2′-*O*-methyl group to the guanine residue at position 10. Melting temperatures were obtained from the maxima of the first derivatives of the melting curves.

3. Results

3.1. Screening of 10–23 DNAzymes with single nucleotide deletions

The contribution of each nucleotide within the catalytic core of the DNAzyme to cleavage activity was established by individually deleting each core nucleotide in a DNAzyme termed DV29 and measuring cleavage activity of the mutants. Most of the deletions resulted in weakly active or inactive DNAzymes, when incubated with their 19-mer target RNA molecules (Fig. 2, black bars). Only DNAzymes with deletions of cytosine at position 7 (C7) and thymine at position 8 (T8) retained high cleavage activity. In the latter case, the performance was even comparable to that of the unmodified DNAzyme.

Experiments with the full-length VR1 transcript revealed similar results (Fig. 2, gray bars). However, the percentage of long target RNA degradation, normalized to cleavage by the unmodified DNAzyme, was lower as compared to that of the short target.

3.2. Activity of deletion mutants against other target RNA molecules

In order to investigate if the observed high cleavage activity of the enzyme with a T8 deletion is sequence specific or if it can be transferred to DNAzyme with other specificity, additional experiments were performed with different RNA substrate molecules. DNAzymes targeting a different site on the VR1 RNA transcript (DV15) and a sequence inside the 5′ non-translated region of the human rhinovirus type 14 RNA

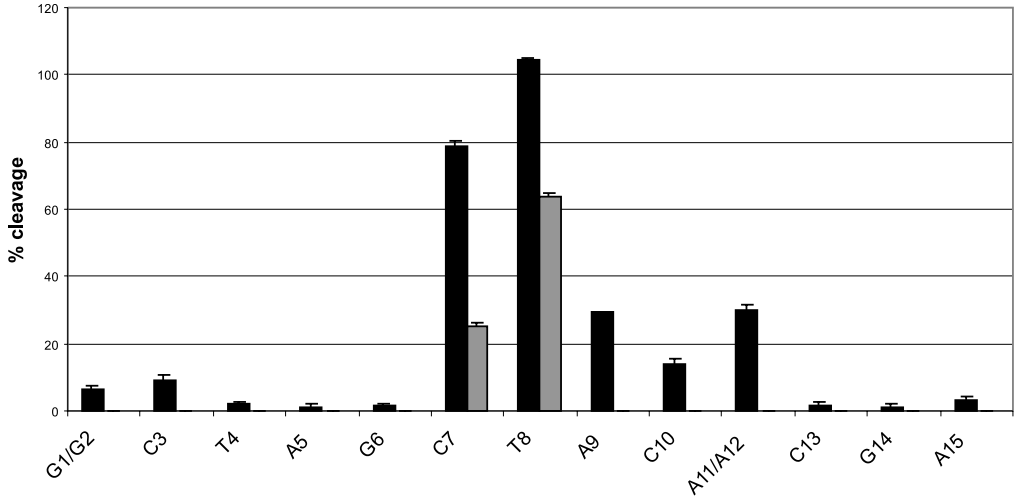

Fig. 2. Target cleavage by DNAzymes with a single deletion in the catalytic core. The percentages of RNA cleavage were normalized to the unmodified DNAzyme. Deletion of G1 and G2 as well as A11 and A12 resulted in the same mutated DNAzyme, respectively. Black bars: short VR1 mRNA target; gray bars: long VR1 mRNA target.

(DH5), respectively, were employed (Fig. 3). In all three cases, cleavage activities of the unmodified DNAzyme and its counterpart with a deletion at position 8 of the catalytic core were found to be comparable. We therefore conclude that it is a general feature of the 10–23 DNAzyme that T8 can be deleted without severe impact on the cleavage activity.

3.3. Kinetic characterization of the deletion mutant

The unmodified DNAzyme has a rate constant of 0.8 min^{-1}, which is in the typical range for 10–23 DNAzymes. For the DNAzyme lacking T8, k_{cat} was only slightly diminished, demonstrating that the deletion of this nucleotide hardly influences the cleavage activity. Kinetic parameters are summarized in Table 2.

3.4. Activity of a DNAzyme with a double deletion

Additionally, a DNAzyme lacking both nucleotides, C7 and T8, was investigated (Fig. 4). Under single turnover conditions with the short target RNA, the double deletion mutant retained significant cleavage activity, comparable to that of the DNAzyme without C7. A closer inspection, however, revealed that the rate constant under multiple turnover conditions is diminished by one order of magnitude (Table 2).

3.5. Ion dependency of the DNAzyme species

To further characterize catalytic activity of the unmodified DNAzyme and the deletion mutants, cleavage reactions with the long target RNA were performed in buffers containing either Mg^{2+} or Ca^{2+} ions at different concentrations, ranging from 1 to 10 mM. Fig. 5 shows that cleavage activities of the wild-type enzyme and the T8 deletion mutant in Ca^{2+} and Mg^{2+} buffers are virtually the same. As described above, the

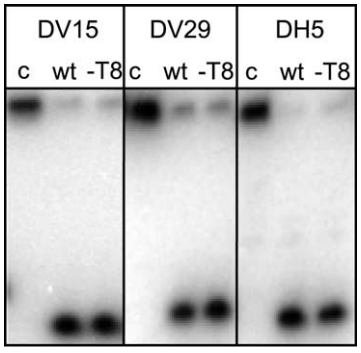

Fig. 3. Cleavage activity of unmodified DNAzymes (DH5, DV15 and DV29) and DNAzymes without T8. The upper band represents the RNA substrate, the lower band corresponds to the 5′ cleavage product. c: control RNA target; wt: wild-type DNAzyme; –T8: DNAzyme lacking T8.

Table 2
Kinetic parameters of the unmodified DNAzyme V29, the DNAzyme lacking T8 and the double deletion mutant C7/T8

DNAzyme	k_{cat} (min^{-1})	K_M (nM)
DV29	0.8 ± 0.1	130 ± 35
DV29 – T8	0.5 ± 0.2	67 ± 15
DV29 – C7/T8	0.05 ± 0.01	14 ± 12

The rate constants (k_{cat}) and K_M values under multiple turnover conditions with the short target RNA are given.

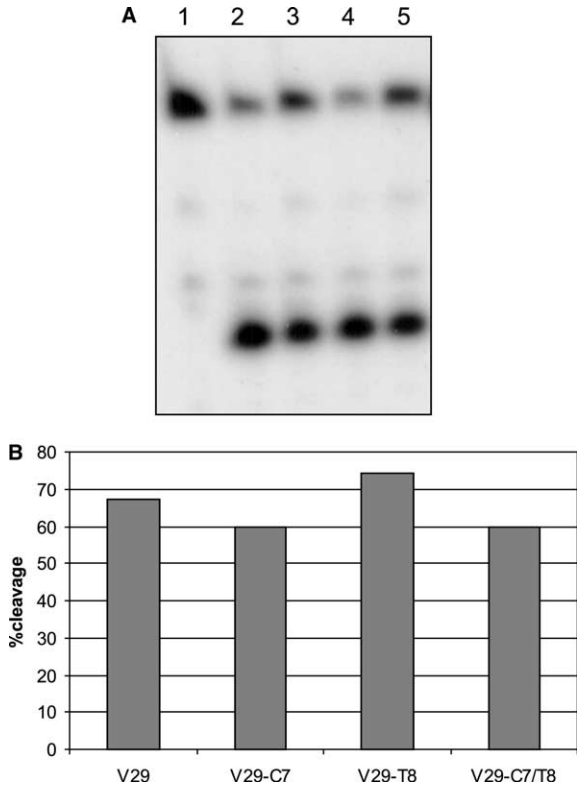

Fig. 4. Cleavage activity of the unmodified DNAzyme and mutated DNAzyme. (A) The upper band represents the short RNA substrate, the lower band corresponds to the 5′ cleavage product. Lane 1: control RNA target V29; lane 2: RNA cleavage by the unmodified DNAzyme; lane 3: RNA cleavage by the DNAzyme lacking C7; lane 4: RNA cleavage by the DNAzyme lacking T8; lane 5: RNA cleavage by the double deletion mutant lacking C7/T8. (B) Diagram presentation of the percentages of target RNA cleavage calculated from the gel above.

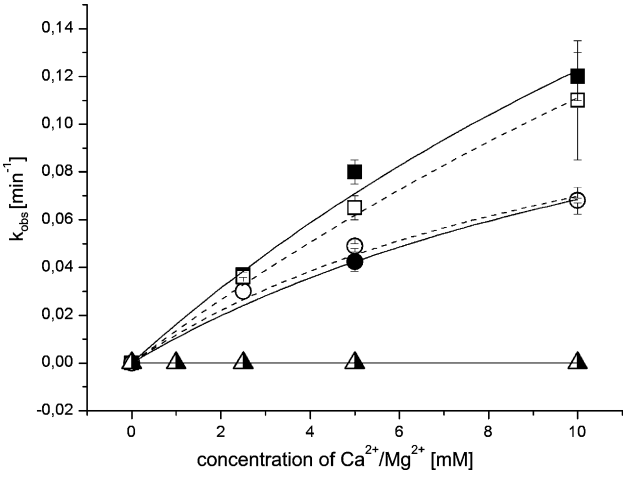

Fig. 5. Observed reaction rates (k_{obs}) of the unmodified DNAzyme DV29, the DNAzyme lacking T8 and the double deletion mutant C7/T8 under saturating enzyme excess conditions with the long target RNA in the presence of different concentrations of Mg^{2+} or Ca^{2+}. Squares, wild type DV29; circles, DV29 T8 deletion mutant; half filled triangles, DV29 double deletion C7/T8 mutant. Solid symbols/lines, buffer containing Mg^{2+} ions; open symbols/dashed lines, buffer containing Ca^{2+} ions at concentrations indicated.

T8 deletion mutant displays a slightly lower activity compared to the unmodified enzyme. The double deletion mutant, however, showed no detectable cleavage of the long substrate molecule with neither Ca^{2+} nor Mg^{2+} ions.

3.6. Melting curves of the DNAzymes

To determine if the deletions in the catalytic core of the DNAzyme might perturb DNAzyme binding to the substrate molecule, melting studies in 10 mM Mg^{2+} reaction buffer were performed. The RNA substrate was rendered resistant to cleavage by the DNAzymes by introducing a 2′-*O*-methyl nucleotide at the cleavage site. Melting temperatures of the unmodified DNAzyme, the T8 deletion mutant and the double deletion species were found to be identical at 54 °C. These results demonstrate that the deletions inside the catalytic core do not alter the binding behavior of the DNAzyme species.

4. Discussion

The type of experiments provided in this communication offers information about the significance of individual nucleotides in the catalytic core for enzymatic activity. This information may aid in the design of catalytically efficient and stable DNAzyme species.

Earlier studies of the hammerhead ribozyme revealed that constructs with stem II shortened to contain 2 bp rather than the conventional 4 bp, which retain essentially unaltered catalytic activity [13]. Furthermore, truncated ribozymes, in which non-critical nucleotides have been replaced by synthetic organic linkers, were enzymatically active [14]. Later on, miniribozymes were obtained by in vitro selection, which are highly active at low physiological Mg^{2+} concentrations [15]. All of these modifications, however, were restricted to the stem-loop II, whereas the region of the hammerhead ribozyme, that forms the catalytic domain, was found to be almost invariant to nucleotide substitutions or deletions [16]. In contrast to the hammerhead ribozymes, we have earlier found the DNAzyme to tolerate a variety of substitutions in the single-stranded core region [11]. Here, we show that even a double deletion mutant lacking two nucleotides, C7 and T8, retains catalytic activity.

Recently, Cruz et al. [17] have performed a series of parallel in vitro selections for DNAzymes cleaving each of the 16 possible combinations of two nucleotides. They rediscovered a large number of active variants of the 8–17 DNAzyme motive, whereas no 10–23 type DNAzyme was obtained. It was argued that the lack of emergence of 10–23 DNAzymes in this and other selection studies is due to lower tolerance of 10–23 DNAzymes to sequence variation, leading to an under-representation of active 10–23 variants compared to 8–17 species in random-sequence libraries [18]. In the light of the results shown here and the relatively high tolerance for substitutions within the catalytic center we reported on earlier [11], however, it might also be possible that the bias against 10–23 variants is rather due to differences in selection conditions between the studies by Santoro and Joyce [1] and Cruz et al.

In previous studies, Sugimoto and co-workers [19–21] have explored the ion and nucleotide dependency of the 10–23 DNAzyme. They found that deletion of four nucleotides within the catalytic loop (A5, G6, C7 and T8) abolished cleavage activity in the presence of Mg^{2+}, but the shortened DNAzyme was highly active in the presence of Ca^{2+}. The deletion mutant T8 that we found in the present study, in contrast, displays almost uncompromised catalytic activity in the presence of either Ca^{2+} or Mg^{2+} ions. Even the double deletion mutant retained cleavage activity in 10 mM Mg^{2+}, when acting on the 19-mer short target sequence. However, almost no cleavage (around 10%) was detected with this mutant, when the target sequence was embedded in a >2500 nucleotides long RNA. A long target molecule like this can be expected to fold into stable structures, making the formation of a productive enzyme–substrate complex more challenging. Also, in the presence of Ca^{2+} ions, cleavage activity of the double deletion mutant was very low. Considering the data reported by Sugimoto et al. and the findings of our study together, it is tempting to speculate that A5 and G6 are directly involved in Mg^{2+}-dependent cleavage activity, but not in Ca^{2+}-dependent catalysis. T8 residue seems to play a minor role, as deletion of this nucleotide has only small effects on catalytic parameters.

Our analysis of the variability of nucleotides in the catalytic core of the DNAzyme has provided a basis for the introduction of modified nucleotides that further enhance resistance against nucleolytic degradation [22]. The deletion study might also be helpful to overcome problems to obtain crystals suitable to solve the three-dimensional structure of the 10–23 DNAzyme in active conformation. In the self-splicing *Tetrahymena* group I intron, deletion of a single nucleotide attenuated the conformational flexibility of a helix so that the mutated ribozyme was readily crystallized and the crystals diffracted to a higher resolution than those of the wild-type RNA [23]. Due to the negligible contribution of T8, one might speculate that this nucleotide does not belong to a highly ordered catalytic domain and obstructs structural analysis because of its flexible nature. The deletion of this thymine might help to stabilize the tertiary structure.

The crystal structures of the 10–23 DNAzyme reported to date are unlikely to represent a catalytically active conformation [24,25]. X-ray analysis revealed a complex formed by two strands of the DNAzyme and two strands of the RNA target. Core nucleotides 2–8 and 15 formed an intermolecular 8 nucleotide long helix between two DNAzyme strands. By using the DNAzyme lacking T8, the self-complementary stretch would be shortened to 6 nucleotides and therefore the formation of the intermolecular helix could be avoided. In this light also the Sugimoto quadruple-deletion mutant lacking C3, T4, A5 and C6 could be a good candidate for crystallization and X-ray diffraction studies. Experiments are on the way to crystallize the single deletion mutant, in order to obtain the first structure of a catalytically active, RNA-cleaving DNAzyme.

Acknowledgments: We thank B. Bieber for technical assistance, J.P. Fürste, M. Vallazza, B. Loll and J. Biesiadka for helpful discussions. This work was financed by the National Foundation for Cancer Research (NFCR, USA) and Fonds der Chemischen Industrie.

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